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(54) Chemically modified protein and production thereof.

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Description

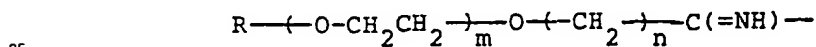
The present invention relates to a biologically active protein having extended blood-circulation life or reduced immunogenicity and to a method of production thereof.

Biologically active proteins are expected to be used as effective drugs. Progress of gene recombination technology has recently allowed the production of such proteins on a large scale. In some cases, however, the biological activity of a protein administered to a living body remains effective only for a short time, due to its extremely rapid elimination in the circulation. In addition, administration of biologically active protein obtained from organisms other than human, such as microorganisms or animals, to human, may result in critical symptoms due to the immune reaction. Therefore, technology development is desired which delays the rate of clearance of the protein and further reduces immunogenicity (antigenicity) thereof with their activity retained.

For the purpose of reducing clearance and immunogenicity, there is a method of modifying biologically active proteins with polyethylene glycol.

15 Polyethylene glycol itself is poorly antigenic, and when combined with an immunogenic protein, it is known to reduce the immunogenicity of the protein. Proteins modified with polyethylene glycol are said to be enhanced in blood-circulation life, and thus to maintain biological activity for a longer time. Means available for coupling polyethylene glycol to a protein include a method using polyethylene glycol methylether and cyanuric chloride (or fluoride) and a method using carboxyl derivatives of polyethylene glycol (US-P-4,179,337). As the former requires a relatively high, alkaline pH at the reaction, it cannot be
20 applied to biologically active proteins which will be inactivated under alkaline conditions. Moreover, the toxicity of cyanuric itself is doubtful. In the latter, coupling agents such as carbodiimide may cause inter- or intra-molecular crosslinking of the protein, furthermore, the destruction of the active conformation of protein may occur due to neutralization of the positively charged groups on the protein by the reaction. In another
25 method, where either alkyl- or alkanoyl-polyethylene glycol aldehyde is introduced into biologically active protein in the presence of a boronic reducing agent, the reducing agent may break the disulfide bond related to the maintenance of the active conformation of protein, and may decrease the biological activity of the protein and insolubilize the protein by changing higher order structure (EP-A-0 154 316).

While working to improve the conventional methods described above, the inventors have now found that the clearance of a biologically active protein from blood-circulation can be reduced by chemically modifying at least one amino groups of the protein with a group expressed by the formula:

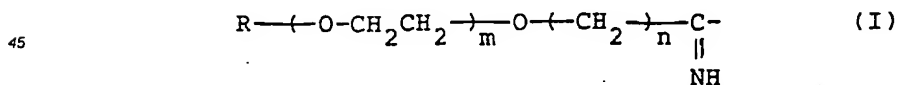


wherein, R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, m represents a positive integer of 500 or less and n represents an integer 1 to 4.

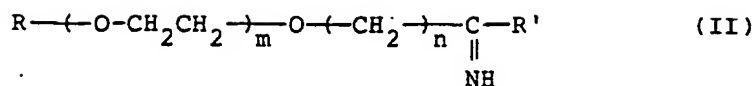
Further studies based on this finding, resulted in the developement of the present invention.

40 The present invention relates to:

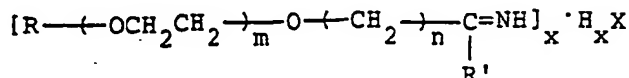
(1) a chemically-modified protein having disulfide bond and a group of the formula:



wherein R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, m represents a positive integer of 500 or less and n represents an integer 1 to 4, the group being bonded to at least one primary amino group of the protein, (2) a method for producing a chemically-modified protein having a group expressed by formula (I), the group being bonded to at least one primary amino group of the protein, which comprises reacting a protein with an imidoester of the formula:



wherein R, m and n have the same meaning as in the previous formula (I), and R' represents a group of -OR'' wherein R'' represents alkyl, aryl or aralkyl, or with a salt of imidoester of the formula:



wherein R, R', m and n have the same meaning as above, x is a positive integer, X is an acid residue and (3) a use of the compound II to produce a chemically-modified protein having disulfide bond and the group of the formula I, the group being bonded to at least one primary amino group of the protein, as a chemically modifying agent.

Proteins which can be used as raw materials in the present invention include those having biological activity; natural proteins derived from animals including humans, from microorganisms or from plants, proteins produced by gene engineering and synthesized protein can be all used as long as they are biologically active. For example, cytokines such as interferons [interferon- α (IFN- α), interferon- β (IFN- β) and interferon- γ (IFN- γ)] and interleukin-2 (IL-2); hormones such as growth hormones and insulin; enzymes such as urokinase, superoxide dismutase (SOD) and asparaginase; and other proteins such as immunoglobulins, trypsin inhibitors, all kind of proteases and peptidases, all kind of cytochromes, islet-activating proteins (IAPs), all kind of inhibitor proteins and neocarzinostatin, can be used. Preferred among such biologically active proteins are various IFNs (rIFN- α , rIFN- β , rIFN- γ) and rIL-2 produced by gene recombination techniques, SOD derived from animals or from microorganisms.

These proteins preferably have a molecular weight of approx. 5,000 to 50,000, especially preferably 10,000 to 30,000.

The primary amino groups of the proteins include N-terminal amino group and ϵ -amino groups of lysine residues.

An alkyl having 1 to 18 carbon atoms represented by R in the above formulas (I) and (II) includes the following moieties: methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, sec-butyl, t-butyl, n-pentyl, isopentyl, n-hexyl, isohexyl, n-heptyl, isohexyl, n-octyl, isooctyl, n-nonyl, isononyl, n-decyl, isodecyl, n-undecyl, n-dodecyl, n-tridecyl, n-tetradecyl, n-pentadecyl, n-hexadecyl, n-heptadecyl and n-octadecyl. Those having 1 to 4 carbon atoms are especially preferred.

The positive integer represented by m in the said formulas is 500 or less, preferably 7 to 150.

The number represented by n in the said formulas is preferably 2 or 3.

The group of formula (I) preferably has a molecular weight of 25,000 or less, more preferably of 350 to 7,000.

The residue R' in formula (II) which represents a group of the formula;

-OR''

wherein, R'' represents an alkyl, an aryl, or an aralkyl.

The alkyls represented by R'' in the above formula preferably has 1 to 8 carbon atoms. Such alkyls include methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, sec-butyl, t-butyl, n-pentyl, n-hexyl, n-heptyl and n-octyl. Those having 1 to 4 carbon atoms are especially preferred.

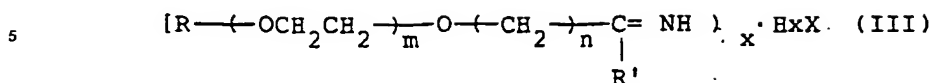
The alkyl may have one or two substituent(s) of chlorine or fluorine.

The aryls represented by R'' include unsubstituted phenyls and phenyls having one or two alkyl(s) of 1 to 3 carbon atoms (e.g. methyl, ethyl, propyl) as substituent(s).

The aralkyls represented by R'' include benzyl, phenethyl and phenylpropyl.

The chemically-modified protein of the present invention can be produced by reacting protein, for example, biologically active protein and an imidoester expressed by formula (II).

Salts expressed by the formula:

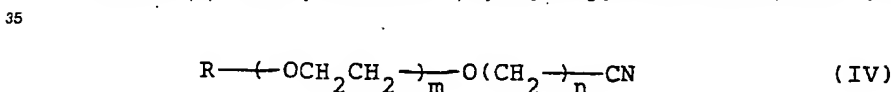


can also be used in place of imido esters expressed by formula (II). (In the formula, R, R', m and n have the same meaning as above. x and X represent a positive integer and an acid residue, respectively.)

In salts expressed by formula (III), x represents the base number of an acid HxX. Any organic or inorganic acid can be used for HxX, however, HCl, CF₃COOH, HClO₄, H₂SO₄, CH₃COOH, CCl₃COOH, and H₃PO₄ are preferred.

When a biologically active protein is modified using compounds (II) or (III), the molar ratio of the compound to the protein can be chosen from the range of 1:1 to 10,000:1. The degree of modification can be optionally chosen by changing either the molar ratio of the protein/compounds (II) or (III), or the concentrations of the protein and compound (II) or (III) in the reaction mixture. Any solvent can be used as long as it does not disturb the reaction and does not inactivate the protein, for example, water, a phosphate buffer, a borate buffer, a Tris buffer and an acetate buffer can be used. An organic solvent which does not inactivate the protein and does not disturb the reaction, such as a lower alkanol (e.g. methanol, ethanol, i-propanol), acetonitrile, dimethyl sulfoxide or dimethylformamide, may be added. In addition, compound (II) or (III) can be added to the reaction mixture directly or after dissolution in either a buffer or an organic solvent as long as the solvent neither disturbs the reaction nor inactivates the protein. It does not matter whether the protein or compound (II) or (III) is first added to the reaction media; simultaneous addition of the protein and compound (II) or (III) is also permitted. The reaction pH can be chosen from a wide range, 3 to 14, but slightly alkaline conditions (pH 7 to 9) is preferable. In addition, the degree of modification of the biologically active protein can be changed by varying the reaction pH. The reaction temperature may be selected within the range in which denaturation of the protein does not occur, but is preferably between 0 and 40 °C. A reaction time of 0.5 to 72 hours, generally 3 to 30 hours, will be sufficient for the reaction. The desired, chemically-modified protein can be obtained by purifying the reaction mixture by conventional methods for purifying proteins such as dialysis, salting-out, ultra filtration, ion exchange chromatography, gel filtration, high performance liquid chromatography or electrophoresis. The degree of modification of amino groups can be calculated, for example, by amino acid analysis after acid hydrolysis.

Imidoester (III) can be synthesized from poly ethylene glycol derivatives expressed by the formula:



wherein R, m and n have the same meanings as above. The following process for the production thereof is simple and preferable.

Thus, the compound (IV) is first dissolved in a dehydrated lower alkanol (HO-R'', wherein R'' has the same meaning as defined above.). The alkanol solution blown with anhydrous acids (e.g. dry hydrogen chloride) is left at -70 °C to +30 °C, preferably at -20 °C. The resulting reaction mixture can be purified using ordinary chemical methods such as concentration, reprecipitation or recrystallization. Thus obtained compound (III) can be stored for a long time under desiccated conditions at low temperature, preferably in a refrigerator or freezer. Compound (II) can be obtained by treating compound (III) with an alkaline compound, e.g. sodium hydroxide or potassium hydroxide.

Polyethylene glycol derivative (IV) can be obtained by the following methods; Polyethylene glycol derivative (IV) (n=3) can be obtained by treatment of the compound of the formula:



wherein R and m have the same meaning as above, except that R does not include hydrogen, with Na, NaH, K or an alkoxide of Na or K, followed by adding bromobutyronitrile [Br(CH₂)₃ CN]; Compound (IV) (n=2) can be obtained by dropping acrylonitrile into compound (V) treated with a small amount of NaH in

an inert solvent, such as benzene. In both cases, the reaction mixture can be purified using ordinary chemical processes such as extraction, concentration, recrystallization, reprecipitation, chromatography or distillation.

The chemically-modified protein of the present invention has the group of formula (I) directly bonded to at least some primary amino groups of the biologically active protein.

The groups of formula (I) should have a molecular weight corresponding to 1 to 10%, more preferably 2 to 8% of the molecular weight of the modified protein.

When the raw protein has amino groups only at its N-terminus, the chemically-modified protein obtained according to the present invention has a group of formula (I) directly bonded to said amino groups.

When the biologically active protein has one or more lysine molecules, the modified protein has the group of formula (I) directly bonded to some of their ϵ -amino groups, preferably 5 to 80% (average), particularly 10 to 50% (average) of the ϵ -amino groups. In this case, the N-terminal amino group may have or may not have the group of formula (I) directly bonded to.

The chemically-modified protein of the present invention has a useful biological activity similar to that of the corresponding unmodified physiologically active protein and thus may be used in drugs.

Compared to the corresponding known unmodified biologically active protein, the present chemically-modified protein is not only reduced in clearance from circulation in living body, thus remaining effective for a longer period, but also lower in both toxicity and antigenicity. Accordingly, it can be used safely for the same purpose and in the same way as well-known biologically active proteins.

The present chemically-modified protein can be administered orally or parenterally to mammals (e.g. monkey, dog, pig, rabbit, mouse, rat, human) in the form of suitable pharmaceutical compositions (e.g. capsules, injection) with the conventional carriers and diluents.

For example, the chemically-modified rIL-2 of the present invention can be used as a preventive or therapeutic agent for tumors; it is administered at a dose similar to that with unmodified rIL-2, i.e. in very small amounts in the form of an injection or capsules.

The chemically-modified rIFN- γ of the present invention, when used as agent for antiviral effect, antitumor effect, cellular growth inhibition, or immunopotentiating activity, is administered to human adults at a dose of 1.0×10^5 to 1.0×10^8 unit/day by intravenous or intramuscular injection.

The chemically-modified rIFN- α A of the present invention, when used as a therapeutic agent for its antitumor effect or antiviral effect, may be administered to patients in 1.0×10^5 to 1.0×10^6 unit/day as amount of rIFN- α A by injection.

The chemically-modified SOD of the present invention, when used as an antiinflammatory agent, may be administered in 1 to 5 mg/kg/day as amount of SOD in the form of an injection or tablets to an animal.

The compound (II) including (III) can be used as a raw material or a starting material for the production of the present chemically modified protein having the group (I).

In the present specification, amino acids, in some cases, are represented by abbreviations based on the IUPAC-IUB Nomenclature (established by the Commission of Biochemical Nomenclature), for example:

Lys: Lysine

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the activity-sustaining effect of the modified proteins in rat blood plasma, as described in Example 1 (iv).

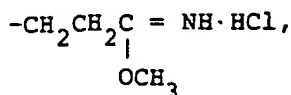
Fig. 2 shows the clearance-retarding effect of the modified proteins in rat blood plasma, as described in Example 2 (iii).

The present invention is hereinafter described in more detail with one reference example and some working examples of its preferred embodiment.

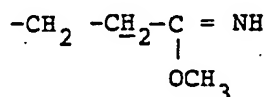
Reference Example 1 Synthesis of polyethylene glycol imido ester

Three grams of polyethylene glycol monomethylether mono- β -cyanoethylether synthesized from polyethylene glycol monomethylether having an average molecular weight of 350 was dissolved in 3 ml of anhydrous methanol. To the resulting solution, dry hydrogen chloride was blown at -20°C to saturation concentration, and the reaction vessel was sealed and left in a freezer for 3 days. After anhydrous ether was added, the solution was cooled again in the freezer. Four hours later, the upper ether layer was decanted; anhydrous ether was added again to the remaining solution. The solution, after being stirred well, was stand in a freezer, yielding solid product about 1 hour later. After the ether was removed, the solid product was washed well with anhydrous ether and cooled in the freezer. After the solid product had settled, the ether

layer was decanted. This process was repeated twice more for thorough washing of solid product. The solid product was dried under reduced pressure in a desiccator containing both phosphorus pentoxide and solid NaOH for 1 hour, yielding imidoester of polyethylene glycol monomethylether having an average molecular weight of 350 (its actual molecular weight is greater than 350 by the weight of



as the polyethylene glycol of average molecular weight of 350 used as the raw material). This product is a syrupy liquid at room temperature. A triplet corresponding to



was detected at the position of $\delta = 2.3$ in $^1\text{H-NMR}$ spectrum (in d_6 -DMSO, 90 MHz) of the product. Absorption corresponding to $-\text{CN}$ disappeared in IR spectrum of the product.

The product was subdivided and sealed in some small containers and stored in a freezer.

Polyethylene glycol mono- β -cyanoethylethers synthesized from polyethylene glycol monomethylethers having average molecular weights of 550, 750, 1,900, and 5,000, respectively, were subjected to the same treatment as above, yielding corresponding imidoester of polyethylene glycol monomethylether.

Example 1 Modification of interleukin-2

(i) Four mg of interleukin-2 (rIL-2), which is obtained by employing *Escherichia coli* DH1/pTF4 (IFO 14299, FERM BP-628) via the method described in Japanese Patent Publication (Unexamined) No.115528/85 or European Patent Publication No. 145,390 was dissolved in 2 ml of 25 mM phosphate buffer solution (pH 7.6) and cooled with ice. With stirring under ice cooling, the resulting solution was added to 17 mg of the imidoester of polyethylene glycol monomethylether (average molecular weight: 550) obtained in Reference Example 1, and then the pH of the reaction mixture was immediately re-adjusted to 7.6 using 0.2N NaOH and reacted for 5 hours. The resulting reaction mixture was applied to a column (0.9 x 50 cm) packed with Sephadex G-25 (Pharmacia AB, Sweden) equilibrated with 25 mM ammonium acetate (pH 6.0), and eluted with the same buffer with monitoring absorption at 280 nm wavelength, yielding approx. 8 ml of a main fraction. The recovery rate of the protein was 60%. Three ml of the fraction, after lyophilization, was hydrolyzed with 6N hydrochloric acid at 110°C in a sealed degassed tube for 24 hours, after that it was subjected to amino acid analysis. The analysis data showed that 2.8 of the eleven Lys residues of rIL-2 had been modified with polyethylene glycol imidoester.

(ii) In the same manner as stated in (i), 24 mg and 57 mg of polyethylene glycol imidoesters obtained by the procedures shown in Reference Example 1 from polyethylene glycol monomethylethers having average molecular weights of 750 and 1,900, respectively, were each added to rIL-2. The amino acid analysis of the reaction product showed that 2.9 and 2.0 of the eleven Lys residues of rIL-2 had been modified with polyethylene glycol imidoesters, respectively.

(iii) Measurements of natural killer (NK) cell inducing activity of the three rIL-2s modified with polyethylene glycol imidoester, obtained in (i) and (ii) gave the values 14,900, 16,500, and 7,180 unit/ml, respectively.

(iv) Approx. 3 mg of each of unmodified rIL-2 and the two rIL-2s modified with polyethylene glycol ester, obtained in (ii), was intravenously administered to rats to measure its biological activity in blood plasma. The following was found; NK cell inducing activity in blood plasma, observed in rats receiving modified rIL-2, remained effective for a longer period than that observed in rats receiving unmodified rIL-2. Lasting of the activity tended to increase with the increase in molecular weight of polyethylene glycol.

The results obtained in the measurements are shown in Fig. 1.

In Fig. 1, Δ - and \square - show chemically-modified rIL-2s of the present invention, obtained in (ii). More specifically, Δ - , \square - , and \circ - show the time-course of killer cell inducing activities in blood plasma after

injection of the modified rIL-2 obtained from polyethylene glycol imidoester having an average molecular weight of 750, of the modified rIL-2 obtained from polyethylene glycol imidoester having an average molecular weight of 1,900, and of the unmodified rIL-2 used as a control, respectively.

5 Example 2 Modification of interferon- γ (rIFN- γ)

- (i) Three ml of rIFN- γ solution (approx. 1.7 mg/ml), obtained by employing *Escherichia coli* 294/pHIT trp 2101 to produce rIFN- γ and by purifying with the use of anti-IFN- γ antibody produced by Mouse B hybridoma gamma 2-11.1 (deposited at C.N.C.M. of Institute Pasteur, France under the deposit number of I-242) via the method described in European Patent Publication No. 110044 or Japanese Patent Publication (unexamined) No. 186995/84, was applied to a column packed with Sephadex G-25 equilibrated with a 20 mM phosphate buffer (pH 7.5) containing 10 mM N-acetyl-cystein and eluted with the same buffer, yielding approx. 4.5 ml of a main protein fraction. With stirring under ice cooling, the resulting IFN- γ fraction was added to 50 mg of imidoester of polyethylene glycol monomethylether (average molecular weight: 750), obtained in Reference Example 1, after that pH of the reaction mixture was immediately re-adjusted to 7.5 using a saturated NaHCO₃ solution. The resulting solution was stirred in a ice bath for more than 4 hours and then stand overnight in a refrigerator. Approx. 3 ml of the supernatant, after removing the resulting precipitates by centrifugation, was applied to a column (1.2 x 45 cm) packed with Sephadex G-75 equilibrated with a 25 mM ammonium acetate buffer solution (pH 6.0) for elution, yielding approx. 5 ml of a main fraction. Reduced form glutathione was added to the fraction to a concentration of 10 mM, yielding a solution containing rIFN- γ modified with polyethylene glycol imidoester. The measurement of rIFN- γ activity (Enzyme-Immunoassay method: EIA method) and antiviral activity of the resulting solution showed the value of 3.4×10^4 ng rIFN- γ /ml and 2.31×10^4 IU/ml, respectively. Part of the supernatant described above was passed through a column (0.9 x 50 cm) packed with Sephadex G-75 equilibrated with 0.1M acetic acid, yielding a protein fraction. After lyophilization, the protein fraction was hydrolyzed with 6N HCl at 110°C in a sealed degassed tube for 24 hours and followed by amino acid analysis. Approx. seven of the twenty Lys residues of the rIFN- γ were found to have been modified.
- (ii) One-hundred mg of imidoester of polyethylene glycol monomethyl ether (average molecular weight: 1,900), obtained in Reference Example 1, and rIFN- γ were treated in the same manner as stated in (i), yielding rIFN- γ modified with polyethylene glycol imidoester. Its rIFN- γ activity (EIA method) and antiviral activity were 3.6×10^4 ng rIFN- γ /ml and 7.7×10^3 IU/ml, respectively. Amino acid analysis showed that approx. two of the twenty Lys residues of the rIFN- γ had been modified.
- (iii) Unmodified rIFN- γ and modified rIFN- γ s obtained in (i) and (ii) were each intravenously administered to rats at doses of 7.90×10^4 , 4.62×10^3 , and 1.54×10^3 IU, respectively. rIFN- γ activities in blood plasma were then measured by EIA method, proving that the rIFN- γ s modified with polyethylene glycol imidoester were clearly slower in clearance than the unmodified one.

Results obtained in the measurements are shown in Fig. 2; - \square -, - Δ -, and - \circ - show time course of changes in plasma rIFN- γ activities after injection of the modified rIFN- γ s obtained in (i) and (ii) and of the unmodified rIFN- γ used as a control, respectively.

Example 3 Modification of interferon- α A (rIFN- α A)

- (i) Five ml of rIFN- α A solution (containing approx. 4 mg protein) obtained via the method described in Japanese Patent Publication (unexamined) No. 79897/1982 or European Patent Publication No.43,980 was dialyzed overnight against 0.05 M phosphate buffer (pH 7.0). To the retentate, 250 mg of the imidoester of polyethylene glycol monomethylether (average molecular weight: 5,000), obtained in Reference Example 1, was added with stirring under ice cooling, after that the pH of the solution was immediately adjusted to 7.5 using 0.2 N NaOH. After stirring for 3.5 hours, the reaction mixture was then applied to a column (0.9 x 50 cm) packed with Sephadex G-75 equilibrated with a 25 mM ammonium acetate buffer solution (pH 6.0) and eluted with the same buffer, yielding a main protein fraction. The resulting fraction was then concentrated using Immersible CX-10 (Millipore Inc., USA), yielding approx. 17 ml of rIFN- α A modified with polyethylene glycol imidoester (protein content: 105 μ g/ml). Amino acid analysis of the acid hydrolysate showed that 3.1 of the eleven Lys residues of the rIFN- α A had been modified. Product activity was found to be 3.0×10^7 IU/ml (EIA method).
- (ii) rIFN- α A was treated with 100 mg of imidoester of polyethylene glycol monomethylether (average molecular weight: 750), obtained in Reference Example 1, in the same manner as stated in (i), yielding approx. 21 ml of solution containing rIFN- α A modified with polyethylene glycol imidoester (protein

content: 107 µg/ml). Amino acid analysis of the product showed that 6.2 of the eleven Lys residues of the rIFN-αA had been modified. Activity measured by EIA method gave the value 7.21×10^6 IU/ml.

When the modified rIFN-αAs obtained in (i) and (ii) were each administered to rats, obvious reduced clearance from blood was found.

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Example 4 Modification of superoxide dismutase (SOD)

(i) 5.5 mg of SOD obtained by employing *Serratia marcescens* ATCC 21074 via the method described in Agricultural and Biological Chemistry, 47, 1537 (1983), Japanese Patent Publication (unexamined) Nos. 29285/1982 and 16685/1983, European Patent Publications No. 45,222 and 70,656, was dissolved in 2 ml of 0.1 M borate buffer (pH 8.7). The resulting solution was reacted overnight with 165 mg of imidoester of polyethylene glycol monomethylether (average molecular weight: 5,000), obtained in Reference Example 1, at pH 8.5 in a cold room overnight. The resulting reaction mixture was then applied to a column (1.8 x 50 cm) packed with Sephadex G-100 and eluted with a 0.05M phosphate buffer (pH 7.0), yielding a main protein fraction, which was then concentrated, yielding approx. 16.5 ml of modified-SOD solution containing 0.282 mg/ml protein.

(ii) *Serratia*-deriving SOD obtained in (i) was added, for reaction, to 330 mg of polyethylene glycol monomethylether imidoester having the same molecular weight as above. The resulting reaction mixture was then treated in the same manner as stated in (i), yielding 22 ml of modified-SOD solution containing 0.226 mg/ml protein.

(iii) Five ml of each of the modified-SOD solutions obtained in (i) and (ii) was dialyzed overnight against distilled water. Each resulting retentate, after lyophilization, was hydrolyzed with 6 N hydrochloric acid at 110°C for 24 hours; 23% and 29% of the SOD Lys residues were found to have been modified, respectively.

(iv) Activity was compared between the modified SODs obtained in (i) and (ii) and unmodified SOD; the residual activity of the modified SOD obtained in (i) and that of the modified SOD obtained in (ii) were 65% and 60% of that of the unmodified SOD, respectively.

Both modified SODs obtained in (i) and (ii) were each found to be slower in clearance from blood and lower in antigenicity compared to unmodified SOD.

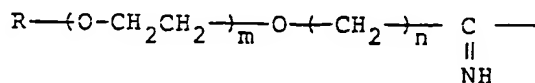
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Claims

Claims for the following Contracting States : DE, GB, FR, IT, NL, SE, CH, LI, BE, LU, GR

1. A chemically modified protein having disulfide bond and a group of the formula:

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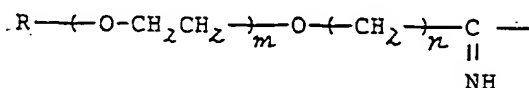
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wherein R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, m represents a positive integer of 500 or less and n represents an integer 1 to 4, the group being bonded to at least one primary amino group of the protein.

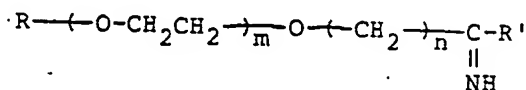
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2. The modified protein according to claim 1, wherein the molecular weight of the group of the formula (I) is 25,000 or less.
3. The modified protein according to claim 1, wherein the protein has molecular weight of from 5,000 to 50,000.
4. A method of producing a chemically modified protein having disulfide bond and a group of the formula:

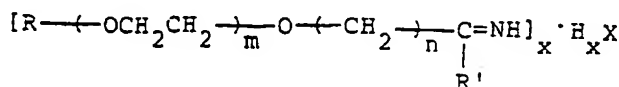
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wherein R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, m represents a positive integer of 500 or less and n represents an integer 1 to 4, the group being bonded to at least one primary amino group of the protein, which comprises reacting a protein with an imidoester of the formula:

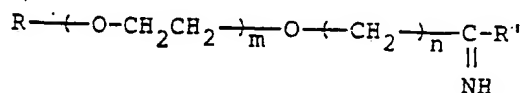


wherein R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, R' represents a group of -OR'' wherein R'' represents alkyl, aryl or aralkyl m represents a positive integer of 500 or less, n represents an integer from 1 to 4, or with a salt of imidoester of the formula.

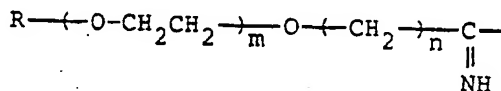


wherein R, R', m and n have the same meaning as above, x is a positive integer, X is an acid residue.

5. A use of a compound expressed by the formula:



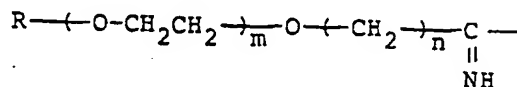
wherein R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, R' represents a group of -OR'' wherein R'' represents alkyl, aryl or aralkyl m represents a positive integer of 500 or less, n represents an integer from 1 to 4, or the salt thereof, to produce a chemically modified protein having disulfide bond and a group of the formula:



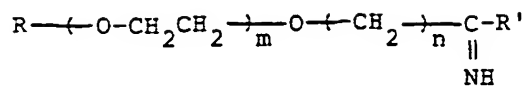
wherein R, m and n have the same meaning as defined above, the group being bonded to at least one primary amino group of the protein, as a chemically modifying agent for protein.

Claims for the following Contracting States : AT, ES

1. A method of producing a chemically modified protein having disulfide bond and a group of the formula:



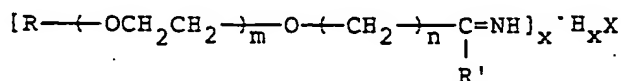
wherein R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, m represents a positive integer of 500 or less and n represents an integer from 1 to 4, an integer 1 to 4, the group being bonded to at least one primary amino group of the protein, which comprises reacting a protein with an imidoester of the formula:



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wherein R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, R' represents a group of -OR'' wherein R'' represents alkyl, aryl or aralkyl, m represents a positive integer of 500 or less, n represents an integer from 1 to 4, or with a salt of imidoester of the formula.

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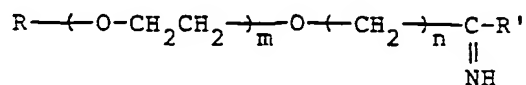
wherein R, R', m and n have the same meaning as above, x is a positive integer, X is an acid residue.

2. The method according to claim 1, wherein R'' is alkyl having 1 to 8 carbon atoms which may have one or two substituent(s) of chlorine or fluorine.

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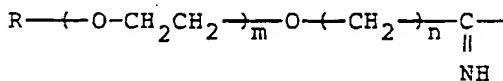
3. A use of a compound expressed by the formula:

25



wherein R represents a hydrogen atom or a alkyl having 1 to 18 carbon atoms, R' represents a group of -OR'' wherein R'' represents alkyl, aryl or aralkyl, m represents a positive integer of 500 or less, n represents an integer from 1 to 4, or the salt thereof, to produce a chemically modified protein having disulfide bond and a group of the formula:

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wherein R, m and n have the same meaning as defined above, the group being bonded to at least one primary amino group of the protein, as a chemically modifying agent for protein.

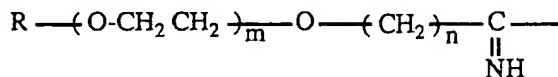
4. A use of the chemically modifying agent according to claim 3, wherein R'' is alkyl having 1 to 8 carbon atoms which may have one or two substituent(s) of chlorine or fluorine.

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Patentansprüche

Patentansprüche für folgende Vertragsstaaten : DE, GB, FR, IT, NL, SE, CH, LI, BE, LU, GR

- 50 1. Ein chemisch modifiziertes Protein mit Disulfidbindung und einer Gruppe der Formel:

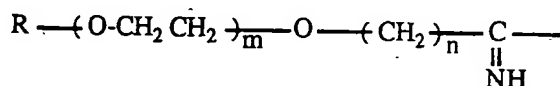


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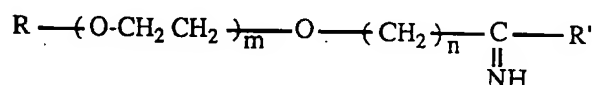
worin R ein Wasserstoffatom oder ein Alkyl mit 1 bis 18 Kohlenstoffatomen darstellt, m eine positive

ganze Zahl von 500 oder weniger darstellt, n eine ganze Zahl 1-4 darstellt und die Gruppe an mindestens eine primäre Aminogruppe des Proteins gebunden ist.

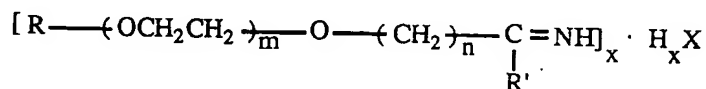
2. Das modifizierte Protein gemäss Anspruch 1, worin das Molekulargewicht der Gruppe der Formel (I) 25,000 oder weniger ist.
3. Das modifizierte Protein gemäss Anspruch 1, worin das Protein ein Molekulargewicht von 5,000 bis 50,000 hat.
4. Ein Verfahren zur Herstellung eines chemisch modifizierten Proteins mit Disulfidbindung und einer Gruppe der Formel:



worin R ein Wasserstoffatom oder ein Alkyl mit 1 bis 18 Kohlenstoffatomen darstellt, m eine positive ganze Zahl von 500 oder weniger darstellt, n eine ganze Zahl 1-4 darstellt und die Gruppe an mindestens eine primäre Aminogruppe des Proteins gebunden ist, welches Reagieren eines Proteins mit einem Imidoester der Formel:

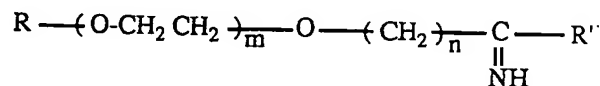


worin R ein Wasserstoffatom oder ein Alkyl mit 1 bis 18 Kohlenstoffatomen darstellt, R' eine Gruppe -OR'' ist, worin R'' Alkyl, Aryl oder Aralkyl darstellt, m eine positive ganze Zahl von 500 oder weniger darstellt, n eine ganze Zahl von 1 bis 4 darstellt, oder mit einem Salz eines Imidoesters der Formel:

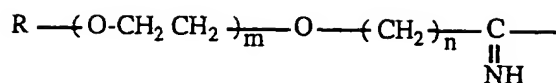


worin R, R', m und n die gleiche Bedeutung wie oben haben, x eine positive ganze Zahl und X ein Säurerest ist, umfasst.

5. Verwendung einer Verbindung, dargestellt durch die Formel:



worin R ein Wasserstoffatom oder ein Alkyl mit 1 bis 18 Kohlenstoffatomen darstellt, R' eine Gruppe -OR'' ist, worin R'' Alkyl, Aryl oder Aralkyl darstellt, m eine positive ganze Zahl von 500 oder weniger darstellt, n eine ganze Zahl von 1 bis 4 darstellt, oder dem Salz davon, zum Herstellen eines chemisch modifizierten Proteins mit Disulfidbindung und einer Gruppe der Formel:



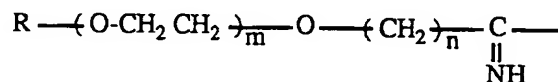
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worin R, m und n die gleiche Bedeutung wie oben haben, die Gruppe an mindestens eine primäre Aminogruppe des Proteins gebunden ist, als ein chemisch modifizierendes Agens für Proteine.

10 Patentansprüche für folgende Vertragsstaaten : AT, ES

1. Ein Verfahren zur Herstellung eines chemisch modifizierten Proteins mit Disulfidbindung und einer Gruppe der Formel:

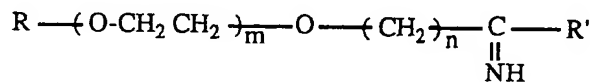
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worin R ein Wasserstoffatom oder ein Alkyl mit 1 bis 18 Kohlenstoffatomen darstellt, m eine positive ganze Zahl von 500 oder weniger darstellt, n eine ganze Zahl 1-4 darstellt und die Gruppe an mindestens eine primäre Aminogruppe des Proteins gebunden ist, welches Reagieren eines Proteins mit einem Imidoester der Formel:

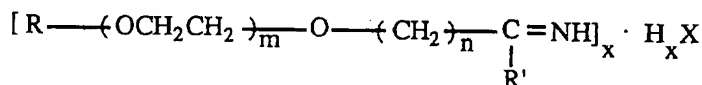
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worin R ein Wasserstoffatom oder ein Alkyl mit 1 bis 18 Kohlenstoffatomen darstellt, R' eine Gruppe -OR'' ist, worin R'' Alkyl, Aryl oder Aralkyl darstellt, m eine positive ganze Zahl von 500 oder weniger darstellt, n eine ganze Zahl von 1 bis 4 darstellt, oder mit einem Salz eines Imidoester mit der Formel:

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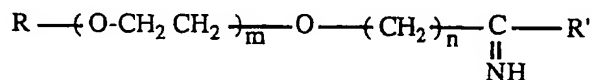
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worin R, R', m und n die gleiche Bedeutung wie oben haben, x eine positive ganze Zahl und X ist ein Säurerest ist, umfasst.

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2. Das Verfahren gemäss Anspruch 1, worin R'' Alkyl mit 1 bis 8 Kohlenstoffatomen ist, das einen oder zwei Substituenten von Chlor oder Flour haben kann.

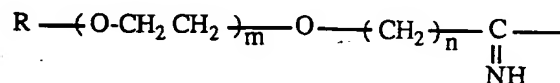
- 50 3. Verwendung einer Verbindung dargestellt durch die Formel:



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worin R ein Wasserstoffatom oder ein Alkyl mit 1 bis 18 Kohlenstoffatomen darstellt, R' eine Gruppe

-OR'' ist, worin R'' Alkyl, Aryl oder Aralkyl darstellt, m eine positive ganze Zahl von 500 oder weniger darstellt, n eine ganze Zahl von 1 bis 4 darstellt, oder dem Salz davon, zum Herstellen eines chemisch modifizierten Proteins mit einer Disulfidbindung und einer Gruppe mit der Formel:



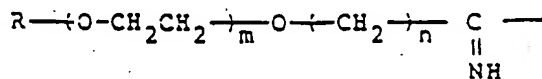
worin R, m und n die gleiche Bedeutung wie oben definiert haben, die Gruppe an mindestens eine primäre Aminogruppe des Proteins gebunden ist, als ein chemisch modifizierendes Agens für Proteine.

4. Verwendung des chemisch modifizierenden Agens gemäss Anspruch 3, worin R'' Alkyl mit 1 bis 8 Kohlenstoffatomen ist, das einen oder zwei Substituenten von Chlor oder Flour haben kann.

Revendications

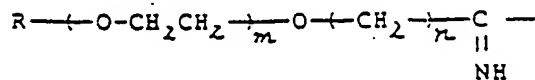
Revendications pour les Etats contractants suivants : DE, GB, FR, IT, NL, SE, CH, LI, BE, LU, GR

1. Protéine chimiquement modifiée ayant une liaison disulfure et un groupe de formule

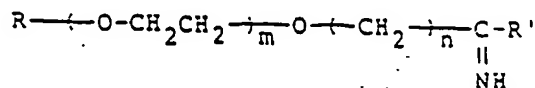


dans laquelle R représente un atome d'hydrogène ou un alkyle ayant de 1 à 18 atomes de carbone, m représente un nombre entier positif égal ou inférieur à 500 et n représente un nombre entier valant de 1 à 4, le groupe étant lié à au moins un groupe amino primaire de la protéine.

2. Protéine modifiée selon la revendication 1, dans laquelle le poids moléculaire du groupe de formule (I) est égal ou inférieur à 25 000.
3. Protéine modifiée selon la revendication 1, dans laquelle la protéine a un poids moléculaire allant de 5000 à 50 000.
4. Procédé de préparation d'une protéine chimiquement modifiée ayant une liaison disulfure et un groupe de formule

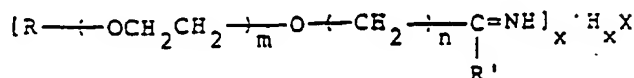


dans laquelle R représente un atome d'hydrogène ou un alkyle ayant de 1 à 18 atomes de carbone, m représente un nombre entier positif égal ou inférieur à 500 et n représente un nombre entier valant de 1 à 4, le groupe étant lié à au moins un groupe amino primaire de la protéine, dans lequel on fait réagir une protéine avec un imidoester de formule:



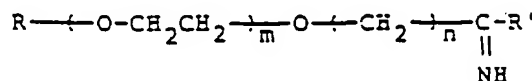
dans laquelle R représente un atome d'hydrogène ou un alkyle ayant de 1 à 18 atomes de carbone, R'

représente un groupe -OR'' où R'' représente un alkyle, aryle ou aralkyle, m représente un nombre entier positif égal ou inférieur à 500, n représente un nombre entier allant de 1 à 4, ou avec un sel d'imidoester de formule



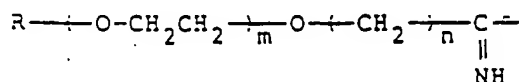
dans laquelle R, R', m et n ont la même signification que ci-dessus, x est un nombre entier positif, X est un résidu acide.

5. Application d'un composé exprimé par la formule



dans laquelle R représente un atome d'hydrogène ou un alkyle ayant de 1 à 18 atomes de carbone, R' représente un groupe -OR'' où R'' représente un alkyle, aryle ou aralkyle, m représente un nombre entier positif égal ou inférieur à 500, n représente un nombre entier allant de 1 à 4, ou de son sel,

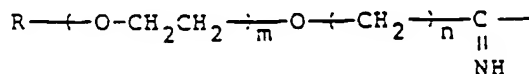
à la préparation d'une protéine chimiquement modifiée ayant une liaison disulfure et un groupe de formule



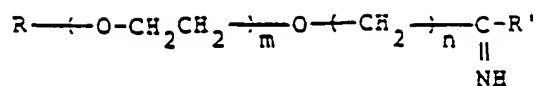
dans laquelle R, m et n ont la même signification que celle qui est définie ci-dessus, le groupe étant lié à au moins un groupe amino primaire de la protéine, comme agent modificateur chimique pour la protéine.

Revendications pour les Etats contractants suivants : AT, ES

1. Procédé de préparation d'une protéine chimiquement modifiée ayant une liaison disulfure et un groupe de formule



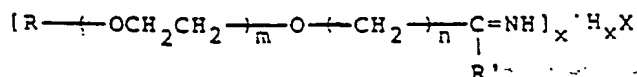
dans laquelle R représente un atome d'hydrogène ou un alkyle ayant de 1 à 18 atomes de carbone, m représente un nombre entier positif égal ou inférieur à 500 et n représente un nombre entier allant de 1 à 4, le groupe étant lié à au moins un groupe amino primaire de la protéine, dans lequel on fait réagir une protéine avec un imidoester de formule:



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dans laquelle R représente un atome d'hydrogène ou un alkyle ayant de 1 à 18 atomes de carbone, R' représente un groupe -OR'' où R'' représente un alkyle, aryle ou aralkyle, m représente un nombre entier positif égal ou inférieur à 500, n représente un nombre entier valant de 1 à 4, ou avec un sel d'imidoester de formule

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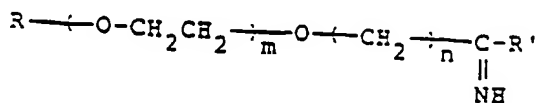
dans laquelle R, R', m et n ont la même signification que ci-dessus, x est un nombre entier positif, X est un résidu acide.

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2. Procédé selon la revendication 1 dans lequel R'' est un alkyle ayant de 1 à 8 atomes de carbone qui peut avoir un ou deux substituant(s) de chlore ou de fluor.

3. Application d'un composé exprimé par la formule

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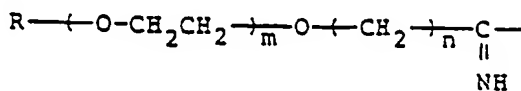
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dans laquelle R représente un atome d'hydrogène ou un alkyle ayant de 1 à 18 atomes de carbone, R' représente un groupe -OR'' où R'' représente un alkyle, aryle ou aralkyle, m représente un nombre entier positif égal ou inférieur à 500, n représente un nombre entier allant de 1 à 4, ou de son sel,

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à la préparation d'une protéine chimiquement modifiée ayant une liaison disulfure et un groupe de formule

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dans laquelle R, m et n ont la même signification que celle qui est définie ci-dessus, le groupe étant lié à au moins un groupe amino primaire de la protéine, comme agent modificateur chimique pour la protéine.

4. Application de l'agent modificateur chimique selon la revendication 3, dans laquelle R'' est un alkyle ayant de 1 à 8 atomes de carbone qui peut avoir un ou deux substituant(s) de chlore ou de fluor.

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Fig. 1

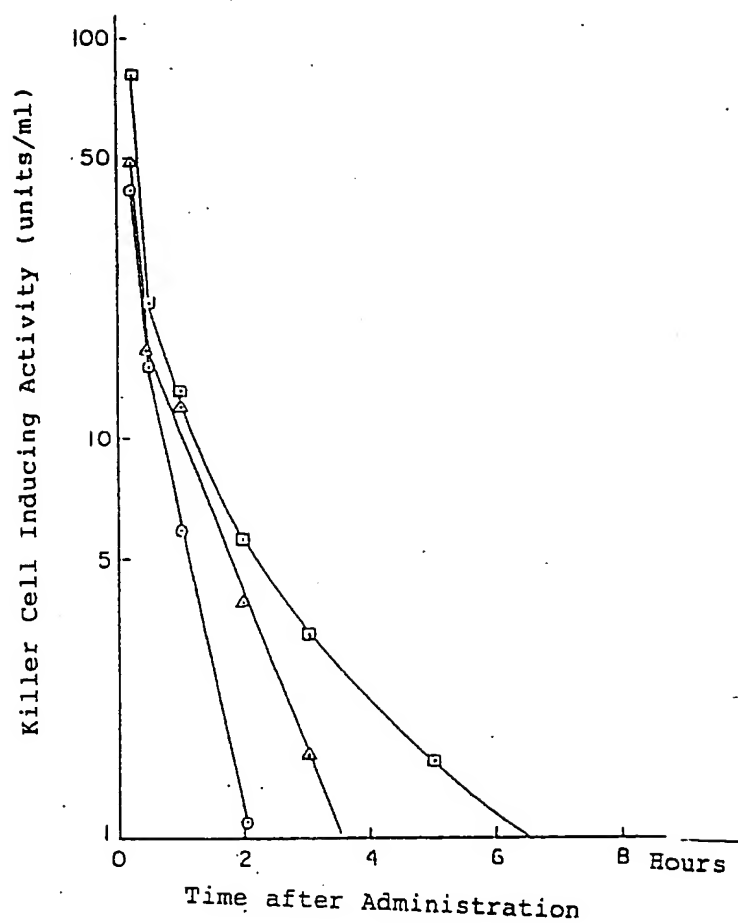


Fig. 2

